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DETECTION AND MOLECULAR CHARACTERIZATION OF A NEW STRAIN AND ISOLATES OF *CANDIDATUS* PHYTOPLASMA AURANTIFOLIA

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ABSTRACT

Witches' broom disease of lime (WBDL) is a lethal disease of unknown origin, identified for the first time in Oman during 1970s and so far killed more than several thousands of small fruited acid lime trees (*Citrus aurantifolia*). WBDL agent *Candidatus* Phytoplasma aurantifolia had been reported earlier and molecularly characterized based on 16S rRNA gene and 16S-23S intergenic spacer region sequence. In the present study, both symptomatic and asymtomatic leaf samples were collected from lime and four other citrus species in five governorates of Oman for detection and identification of different isolates or strains of *P. aurantifolia*. Both PCR amplification of 16S rRNA gene and 16S-23S spacer region, performed utilizing three pairs of primers(R16F2n/R2, AdF1/R1 and P1/P7) and transmission electron microscopy of phloem vessel elements confirmed phytoplasmal infection in only symptomatic plants. AdF1/R1 primed PCR products of *C. limetta, C. medica, C. lemon* and seven samples of *C. aurantifolia* were sequenced each with 1.706 kb. The results of the present investigation had led to identification of five isolates among the ten and a new strain of the causal agent of WBDL on the basis of the two phylogenetic trees constructed by parsimony analysis of 16S rRNA gene plus 16S-23S spacer region from the ten isolates of *P. aurantifolia* and phytoplasma members from 16SrII group with the support of sequence comparison and similarity. This is the first report showing electron microscopy, WB symptoms and PCR detection of natural infection of WBDL in *C. medica* and *C. lemon* plants.

KEYWORDS: Detection, Molecular Characterization, Isolates, Candidatus, Phytoplasma Aurantifolia, Lemon

INTRODUCTION

Lime (*Citrus aurantifolia* Swingle) is widely grown in tropical and subtropical countries of the world including Oman for many generations and is associated with the national heritage of the country. The "unknown origin disease" Witches Broom Disease of Lime (WBDL) was first discovered in Oman by Walker and Bridge in 1978 and described by Bove in 1986. It has so far killed more than half a million of small fruited acid lime trees. The new lime trees whenever planted are also under threat of this disease, which although associated with Phytoplasmas, their exact role is yet to be discovered. Since the outbreak of this serious and destructive disease, a few researches and studies were attempted towards descriptions of the disease and symptoms, detection and identification of causal agent by ELISA, PCR and electron microscopy, host range *in vitro*, sequencing of 16S rRNA gene and phylogenetic classification (Garnier, *et al*, 1991; Moghal, *et al*, 1993; Zreik, *et al*, 1995; Al-Sadi *et al*, 2012).

Over a decade, the application of molecular techniques like PCR has served as the most reliable approach in identifying, characterizing and establishing phylogenetic relationships of the strains of phytoplasmas that are either virulent or mild or avirulent, causing witches' broom like symptoms in different plant species *viz. Cassia italic* (Al-Saady et. al,

2008), Sesame (*Sesamum indicum*) (Bove, 1986; Khan *et al*. 2007), Arabian jasmine (*Jasminum sambac*) (Al-Zadjali *et al*, 2007), Beach Naupaka(*Scaevola taccada*) (Al-Zadjali et al, 2012) and alfalfa(Khan *et al*, 2002; Al-Zadajali *et al*, 2007) in Oman. The phylogenetic relationship among the phytoplasma strains is based on the sequence of 16S rRNA gene sequences among which several subtaxa of *Candidatus* Phytoplasma share <97.5% similarity between each other (IRPCM, 2004).

MATERIALS AND METHODS

Collection of Samples

Two surveys were carried out in five Governorates of Oman during April 2006 and August 2007 which covered 150 farms from where in total 60 plant leaf samples were collected from the five citrus *spp*. based on phytoplasma like symptoms and asymptomatic plant specimens were also sampled from the apparently uninfected lime trees surrounded by witches broom infected ones (Table 1).

Transmission Electron Microscopy

Small pieces of leaf midrib and petiole tissues of symptomatic and asymtomatic samples were collected (Table 2) which were cut into 2 mm sections and fixed immediately with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) prior to their use at Utsunomiya University, Japan, where they were stored at 4^{o}C. The specimens were subsequently post-fixed in 1% osmium tetraoxide in the same buffer for 1 h, then washed twice(10 min each) in 0.1 M cacodylate buffer. Samples were then dehydrated by passing through an ethanol series and embedded in resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined through Hitachi H-700 electron microscope.

Nucleic Acid (DNA) Extraction and PCR Amplification

DNA was extracted from all the samples including asymtomatic ones using a DNeasy Plant Mini Kit (QIAGEN, Maryland, USA) according to manufacturer's instructions. Three primer pairs, R16F2n/R2 (Lee et al, 1993), P1/P7 (Deng & Hiruki, 1991; Schneider et al, 1995) and AdF1/R1(Al-Zadjali et al, 2007) were used to amplify the 16S rRNA gene and the 16S-23S intergenic spacer region. Amplification was performed by direct and nested PCR in an automated thermo cycler (Minicycler, MJ Research Inc, USA) using Taq DNA polymerase with the following PCR conditions for the P1/P7 primer pair: 40 cycles at 94^{o}C for 2 min in the first step, denaturation at 94^{o}C for 1 min, annealing at 52^{o}C for 30 s, extension at 72^{o}C for 30 s and final extension at 72^{o}C for 2 min. For the AdF1/R1 and R16F2n/R2 primer pairs the following conditions were used: 35 cycles at 94^{o}C for 4 min in the first step, denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 2 min, extension at 72^{o}C for 3 min and final extension at 72^{o}C for 5 min.

Each PCR reaction mixture (50 µl) contained 25 ng of total nucleic acid plus 20 pmol of each primer, 1.0 unit of Taq DNA polymerase and a final concentration of 0.2 mM of dNTP, 2.0 mM $MgCl_2$ and 10x PCR buffer. Products of direct PCR primed by P1/P7 were diluted in sterilized distilled water 50 times (1:50) and 1 µl samples were used as template DNA in nested PCR primed by AdF1/R1.

Cloning of PCR Products

Nested PCR products of 1.7 kb fragments of 16S rRNA gene and 16S-23S intergenic spacer region were cloned using a cloning kit(pGM-T Easy Victor System II; Promega, Madison,WI, USA) according to the manufacturer's instructions.

Recombinant plasmids were screened by blue/white colour screening on indicator plates and PCR amplified using AdF1/R1 primer pair.

Sequencing and Phylogenetic Analysis

Cloned plasmids were extracted from selected colony, then purified and sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosytems. Foster City, CA, USA). The sequence data of the new strain and isolates of Phytoplasma reported in this study were submitted to the DNA Data Bank of Japan (DDBJ) with accession numbers assigned as AB295056, AB295057, AB295058, AB295059, AB295060 and AB295061. Sequence similarity and putative RFLP of new sequences were evaluated by comparing the sequence homolog and restriction sites of 102 restriction enzymes with the reported sequence of *Candidatu*s Phytoplasma aurantifolia-LWB (GeneBank: U15442) based on 1.7 kb sequence using Genetyx software(GENETYX Co, Tokyo, Japan); the homology search option and the percentage homology results were manually added to the similarity analysis table after alignment. The phytoplasmal 16S rRNA gene and 16S-23S intergenic spacer region sequences of 1.7 kb in size, primed by AdF1/R1 from new isolates and the reported LWB phytoplasma were used to generate a phylogenetic tree based on the neighbour-joining method using CLUSTAL W (Thompson et al, 1994) and the Mac Vector package(Oxford Molecular Ltd, Oxford, UK). A sequence phylogenetic tree was constructed using the TREE VIEW program by performing bootstrap analyses (1000 bootstraps) of the data with Laser Gene software (DNASTAR, Madison, WI, USA). The sequences were then visually inspected for logical placement. The extra phylogenetic tree was generated from the strains belonging to the 16Sr II group including the new isolates to examine the phylogenetic relationship of the new sequences among other members of the 16Sr II group based on 1.7 kb sequences.

RESULTS

Symptomtology

The disease symptoms were characterized by their appearance and progressive development of witches' broom from their initiation at one part of the tree extending throughout the canopy. The leaves on the witches' broom are small to very small and pale green to yellow after short time which later dry up but remain attached to shoots for some time and then eventually fall. Very few abnormal flowers and small fruits are formed on witches' brooms. Diseased tree often takes 3 to 5 years to die totally from the first appearance of witches' broom symptoms (Figure). The collected samples of lime (*Citrus aurantifolia*), sweet rough lemon (*C. limetta*), citron (*C. medica*) and lemon (*C. lemon*) had typical witches' broom symptoms.

Transmission Electron Microscopy

The presence of phytoplasma-like structures in phloem elements of symptomatic lime, sweet rough lemon, citron and lemon plants and the absent of these structures in healthy lime indicated the phytoplasma infection in these symptomatic plants (Figures 1-4)

PCR Amplification

A fragment of 1.7 kb product of 16S rRNA plus spacer region was successfully amplified by using AdF1/R1 primer pair from five witches' broom infected limes collected from five Governorates of Oman symptomatic sweet rough lemon and lemon plants (Table 2). However, no fragments were detected in sweet lime samples which showed slight yellowing symptoms and in lime samples which were symptomless (Figure 3).

Sequence Similarity

The sequence similarity percentage among the 10 samples and LWB (Table 2) revealed that samples no. 20 (*C. limetta*), 24(*C. aurantium*) and 34(*C. aurantifolia*) had 100% similarity to each other based on 1.7 kb sequence of the 16S gene and spacer region. Likewise, sample nos of *C. aurantifolia.* 8, 22, 37 and 38 were found to be 100% similar to each other. However, the sample no. 27(*C. lemon*) showed 99.414% similarity to sample nos. 8 and 22 and 99.063% to WBL sample (Table 3).

Phylogenetic Analyses

Based on 16S rRNA gene plus 16S-23S spacer region sequences of *Candidatus* Phytoplasma aurantifolia strains, phylogenetic analysis indicated that samples Ad34(lime from Dhahirah), Ad20(sweet rough lemon from Dakhliya) and Ad24(sour orange from Batinah) could be the root of this phylogenetic tree. Samples Ad8 and Ad22 had shown similar distance from the root of the tree. Similarly AD 37 and AD 38 had located at the same distance farther than AD8 and AD22. On the contrary Ad27 from Batinah was located too far from all other samples (Figure 5). Figure 5 demonstrated the relationship between the 10 new sequences of *Candidatus* P. aurantifolia and other phytoplasmas belonging to 16SrII group. This phylogenetic analysis tree revealed that Ad27 was different strain of *Candidatus* P aurantifolia, as it appeared as a separate branch (Figures 4 & 5).

DISCUSSIONS

Electron microscopy and PCR were for the first time applied to visualize and detect phytoplasmas associated with naturally infected lemon (*Citrus lemon*), sweet rough lemon (*C. limetta*) and sour orange (*C. aurantium*).

Sweet lime is an important citrus crop widely grown in Oman, which has never shown any type of phytoplasma infection symptoms in the field throughout our surveys since 1990's until to-date (Moghal et al, 1993, Al-Zadjali et al, 2007). Sweet lime (*C. limettioides*) is a hybrid between a lime (*C. aurantifolia*) susceptible to WBDL and sweet orange (*C. sinensis*) resistant to WBDL which was confirmed through graft infection tests earlier (Bove' et al, 1996). Considering this, in the present study all the three samples of sweet limes were found to show only slight yellowing symptom but they failed to amplify any bands of phytoplasmal 16S rRNA gene in direct and nested PCR amplification tests. This confirmed the resistance of sweet lime against *Ca*. Phytoplasma aurantifplia which offers an opportunity for its use as parent in transferring WBDL resistance to limes through genetic engineering.

Reported sequence of LWB had shown 1705 bp fragment size with AdF1/R1 primer pair amplification through putative alighment whereas in the present study all the ten isolates had clearly shown 1706 bp size with AdF1/R1 primer pair amplified fragment. Therefore 1706 bp size sequence has been proposed for this portion of 16S rRNA gene plus 16S-23S spacer region with the inclusion of extra Adenine (A_{1576}).

Mutation is possible in rDNA of any microorganism due to several factors (Lodish *et al*, 2000). The 16S rRNA gene and 16S-23S interagency spacer region are non-coding regions of phytoplasma rDNA. These changes in non coding regions, as translation of Adenine (A) to Guanine (G) and G to A (Purines), or from Thymine (T) to Cytocine (C) and C to T (Pyrimidines) are due to common mutations. Such changes from Purimes (A or G) to Pyrimidines (T or C) and the vice versa would affect the amino acids suppression. Since 16Sr RNA gene sequence is the basis of phytoplasma classification, the current study proposes new 6 sequences in 16SII group from the 10 samples studied.

In the present investigations, a new strain of *Candidatus* Phytoplasma aurantifolia asstrain Ad27 Lemon (Acc. No. AB295060) and 5 isolates namely Ad20 Limetta (Acc. No. AB295057), Ad8 (AB295056), isolate Ad37(AB295059), isolate Ad21(AB295058) and isolate Ad32(AB295061) were identified. Even though LWB phytoplasma has been detected, identified and molecularly characterized in 1995 by Zreik and her team, the current study revealed that the isolate Ad20Limetta had a wider host range and more widely spread than other isolates as this isolate was detected in 3 different citrus species, *C. limetta*, *C. aurantifolia* and *C. aurantium* collected from 3 different governorates of Oman and had been found to be the root of other isolates and strains.

Experimentally WBDL was successfully transmitted to healthy lime and periwinkle (*Catharanthus ruseus*) plants through grafting and parasitic plants (dodder, *Cascutta sp*.). However, transmission of WBDL through insect vectors has not yet been confirmed except the fact that leaf hopper *Hishomonas phycitis*, was often found in association with WBDL in fields and had shown positive result in ELISA and PCR detections because of which *Hishomonas phycitis* was considered as a candidate vector of WBDL phytoplasma. In the restriction sites analysis during present investigations, it was found that all the five new isolates were indistinguishable not only from each other but also from the reported strain Candidatus Phytoplama aurantifolia, which altogether were distinct from the strain Ad27 Lemon in 3 restrictions sites. All these isolates were found to have 6 new different sequences.

CONCLUSIONS

The results of the present investigations clearly demonstrated electron microscopy of phytoplasma bodies in phloem vessel elements of naturally witches' broom infected lemon and citron for the first time. The study further discovered a new phytoplasma strain in citrus plants such as *Citrus lemon (Candidatus* Phytoplasma lemon) and five new isolates of *Candidatus* Phytoplasma aurantifolia in *C. aurantifolia*.

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APPENDICES

Table 1: Plant Samples and Their Collection Areas in Oman

Name	Botanical Name	No. of Samples and Location Area
Symptomless lime	*Citrus aurantifolia*	25(5D, 5B, 5S, 5Dh, 5M)
Lime	*Citrus aurantifolia*	25(5D, 5B, 5S, 5Dh, 5M)
Lemon	*Citrus lemon*	2(Rumais B)
Sweet lime	*Citrus limtiodes*	3(B,!D,!Dh)
Citron	*Citrus medica*	2(B Rumais & Flaj Al Muraq D)
Sweet rough lemon	*Citrus limetta*	3(B Rumis, Bahla D, Rudah S)

†(B) Batinah,(D) Dakhliya,(Dh) Dhahirah,(M) Musandam and(S) Sharqiyah Governorates.

Table 2: Plant Species Examined by Transmission Electron Microscopy

Name	Botanical Name	Family
Healthy lime	*Citrus aurantifolia*	Rutaceae
Lime	*Citrus aurantifolia*	Rutaceae
Sweet rough lemon	*Citrus limetta*	Rutaceae
Citron	*Citrus medica*	Rutaceae
Lemon	*Citrus lemon*	Rutaceae

Table 3: Sequence Similarity Analysis of LWB in Comparison with the New 10 Isolates

			Similarity (%)								
Samples	**LWB**	**8**	**20**	**21**	**22**	**24**	**27**	**32**	**34**	**37**	**38**
LWB	**100**										
8	99.649	100									
20	99.707	99.941	100								
21	99.649	99.883	99.941	100							
22	99.649	100	99.941	99.883	100						
24	99.707	99.941	100	99.941	99.941	100					
27	99.063	99.414	99.355	99.297	99.414	99.355	100				
32	99.590	99.941	99.883	99.824	99.941	99.883	99.357	100			
34	99.707	99.941	100	99.941	99.941	100	99.355	99.883	100		
37	99532	99.706	99.824	99.766	99.706	99.824	99.179	99.707	99.824	100	
38	99.532	99.706	99.824	99.766	99.706	99.824	99.179	99.707	99.824	100	100

LWB- reported Lime Witches' broom *Candidatus.* P. *aurantifolia*(Acc. No. U 15442),(8, 37).

WB -infected Lime(AB295056 & AB295059) from Batinah Governorate,(20).

WB- infected Sweet rough lemon(AB295057),(21) WB infected Lime from Dakhliya(AB295058),(22)

WB- infected Lime from Musandam,(24) WB infected Citron,(27) WB infected Lemon(AB295060),(32)

WB infected Lime from Sharqiya(AB295061),(34) WB infected Lime from Dhahira(AB295057).

Figure 1: Symptoms of Witches' Broom Disease of Lime

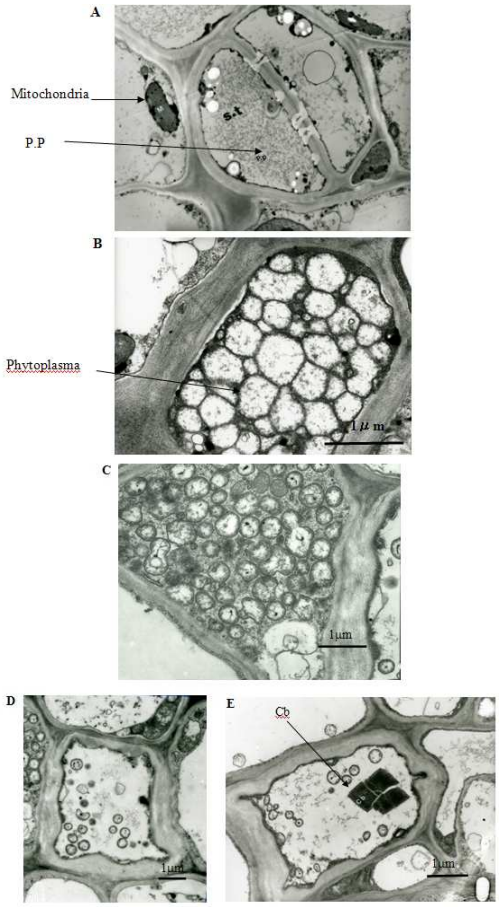

Figure 2: Transverse Section (TS) of Healthy and Witches' Broom Infected Vessel Elements

- Phloem vessel element of healthy lime plant (*Citrus aurantifolia*) showing sieve tube(S.t.) with P protein(P.p) and absence of any strange bodies.
- Sieve tube of WB infected lime, packed with phytoplasma bodies.
- Phloem vessel element of WB infected sour orange(*C.aurantium*) showing a high number of the phytoplasma.
- Phloem vessel element of WB infected Lemon(*C. lemon*) shoeing low number of the phytoplasma.
- Phloem vessel element of WB infected sweet rough lemon(*C. limetta*) showing a low number of the phytoplasma and crystal-like bodies(Cb).

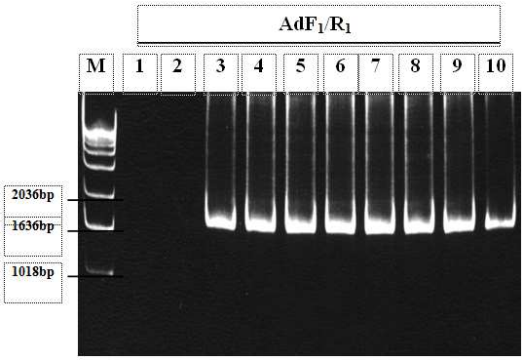

Figure 3: Nested PCR Amplification Products of WB Symptomatic, Asymptomatic and Healthy Citrus Samples

Polyacrylamide gel 5% showing PCR amplification of phytoplasmal 16S plus 16S-23S rDNA sequence.(1) healthy lime,(2) sweet lemon showing slight yellowing,(3) lemon with yellowing and die back,(4) sour orange with witches' broom,(5) sweet rough lemon with witches' broom and(6-10) witches' broom-infected lime samples represents five Governorates of Oman (Table 1). Amplification was performed using an AdF1/R1 primer pair.(M) 1 kb DNA ladder is shown in line one (manufactured by Invitrogen).

Figures

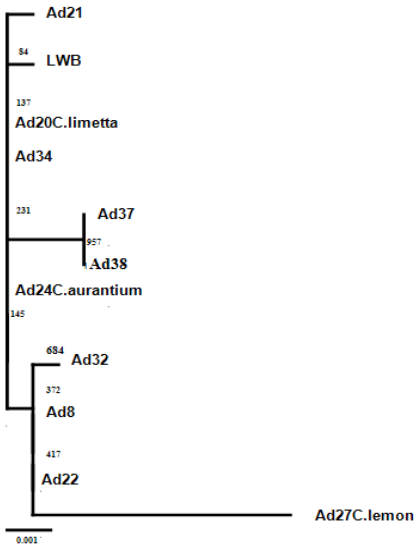

Figure 4: Phylogenetic Analysis Tree of the 10 New Isolates with LWB

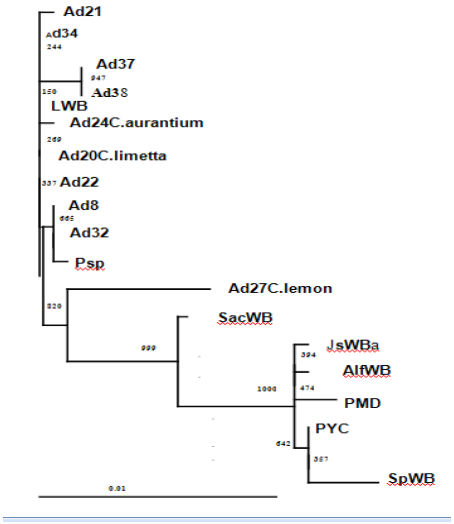

Figure 5: Phylogenetic Relationship of the New 10 Isolates among 16srII Group Phytoplasma Strains